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Journal of Chromatography A, 918 (2001) 99–112

JOURNAL OF CHROMATOGRAPHY A

www.elsevier.com/locate/chroma

Critical study of and improvements in chromatographic methods for the analysis of type B trichothecenes ☆

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Received 20 November 2000; received in revised form 14 February 2001; accepted 26 February 2001

Abstract

Various analytical methods used in the analysis of type B trichothecenes (deoxynivalenol, nivalenol, 3- and 15-acetyldeoxynivalenol) in cereals were compared and optimised in this work. These methods use either GC–electron-capture detection (ECD) of trimethylsilyl, trifluoroacetyl and heptafluorobutyryl derivatives or HPLC with UV or photodiode array detection of analytes. A new HPLC procedure using fluorescence detection prior derivatisation with coumarin-3-carbonyl chloride has been also tested. Five extraction solvents and two solid-phase extraction cartridges (silica, Florisil) plus a especial clean-up column (MycoSep 225) were compared in order to obtain the best recovery of the mycotoxins with minimal presence of coextractives in the chromatograms. The chosen extraction solvent was a mixture of acetonitrile–water (84:16, v/v). The MycoSep 225 column was chosen as the best alternative for clean-up of grain samples. For GC–ECD analysis, derivatisation of analytes with heptafluorobutyric anhydride prior the final determination was chosen as the most suitable procedure. HPLC–photodiode array (at 221 nm) analysis was more suitable for determination of type B trichothecenes than HPLC of the fluorescent coumarin-3-carbonyl derivatives. Recoveries obtained in spiked corn, rice and wheat are reported. The utility of the proposed methodology was assayed in cereal cultures of various *Fusarium* strains.

Keywords: Food analysis; Cereals; Mycotoxins; Trichothecenes; Deoxynivalenol; Nivalenol; Sesquiterpenoids

1. Introduction

Trichothecenes are a family of closely related sesquiterpenoids. Most of them have a double bound at position C-9,10, a 12,13 epoxide ring and a variable number of hydroxyl and acetoxy groups. This group of mycotoxins is produced by several genera of fungi [1], but cereals contaminated by various *Fusarium* species are the main source of nonmacrocyclic trichothecenes in food and feedstuffs [2–5].

Although the number of characterised trichothecenes is large, only a few of these have been detected so far in naturally contaminated cereals and commodities, mainly belonging to both type A or type B trichothecenes. Type B trichothecenes are characterised by a carbonyl group at C-8 and in-

☆ Presented at the 29th Scientific Meeting of the Spanish Group of Chromatography and Related Techniques, Alcalá de Henares (Madrid), 12–14 July 2000

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0021-9673/01/$ – see front matter
PII: S0021-9673(01)00704-X

clude, among others, deoxynivalenol (DON), nivalenol (NIV), and their derivatives.

Analysis of contaminants in grains and plant products is not simple. Many variables must be carefully controlled, such as suitable sampling from large quantities of starting material, and handling, storage, preparation and subsampling of initial samples to ensure that analysis is carried out on a truly representative sample of test material [6]. Analysis is further complicated by possible heterogeneity of the starting material and the presence of contaminants in the storage or delivery systems. On the other hand, during the analysis, variables such as precision, accuracy and detection limit need to be assessed to assure that quantities measured are representative of the starting material and that analytical errors are minimised [7,8].

Various combinations of solvents have been used for extraction of different substrates. In the original procedures, samples were extracted with methanol–water (50:50, v/v) [9–11]; in a later version, the ratio of these solvents was changed to 70:30 (v/v) [12,13]. Acetonitrile–water in somewhat variable ratios, has been applied by a number of laboratories. Acetonitrile–water (84:16, v/v) [14,15] is probably the extraction medium most extensively used for trichothecene analysis today [16,17], although Tanaka et al. [18] found that highest recovery of NIV and DON was obtained with acetonitrile–water (75:25, v/v).

Various clean-up procedures applicable for trichothecene analysis have been published. Most of them include adsorption chromatography on Florisil [19,20], silica gel [19,21] or charcoal–alumina column [22,23]. Alternatively, gel permeation chromatography has been employed [21,24].

A variety of chromatographic methods have been described for determination of type B trichothecenes. The first methods were based on thin-layer chromatography; trichothecenes were visualised by various reagents, such as aluminium chloride [25,26]. Later, gas chromatography (GC) methods based on electron-capture (ECD) [10,15,20,27–30] or mass spectrometry (MS) detection [12,31–34] were established. High-performance liquid chromatography (HPLC) [28,35], supercritical fluid chromatography [36] and enzyme-linked immunosorbent assay [37,38] are other analytical techniques used for determination of trichothecenes.

Because of the relevance of these mycotoxins in food and feed, the aim of this work was to perform a comparative study of different chromatographic analytical methods to determine these metabolites in a great number of cereal samples. Owing to the large variety of existing methods the evaluation and discussion of them is necessary to choose the most suitable optimised methodology. A new derivatisation procedure to give fluorescent derivatives of these metabolites, which was previously applied to type A trichothecenes, has been tested as well.

2. Experimental

2.1. Chemicals and reagents

Trichothecene mycotoxins, including DON, NIV, 15-acetyldeoxynivalenol (15-AcDON), and 3-acetyldeoxynivalenol (3-AcDON) were supplied by Sigma (Steinheim, Germany). They were individually dissolved in acetonitrile at a concentration of 1 mg/ml and stored at −20°C and brought to room temperature before use.

Regarding GC derivatisation reagents, 1-(trimethylsilyl)imidazole (TMSI), trimethylchlorosilane (TMCS), *N*,*O*-bis(trimethylsilyl)acetamide (BSA) and heptafluorobutyric anhydride (HFBA) were obtained from Fluka (Buchs, Switzerland) and trifluoroacetic anhydride (TFAA) and 4-dimethylaminopyridine (DMAP) from Sigma. For HPLC–fluorescence detection derivatisation coumarin-3-carboxylic acid and thionyl chloride were purchased from Aldrich (Gillingham, UK).

All solvents were analytical or HPLC grade and were obtained from Merck (Darmstadt, Germany). Water was obtained from Waters Milli-Q system (Waters, Milford, MA, USA).

2.2. Fungi and samples

Two type B trichothecenes-producing strains of *Fusarium graminearum* (FG1 and FG6) and one *F. culmorum* strain (FCu1) were used to inoculate cereal samples. These strains are held at the collection of the Department of Microbiology of the University of Valencia and maintained in Potato-Dextrose Agar (PDA).

Cereal (corn, rice or wheat) samples were pur-

chased commercially and used without grinding. They were tested for absence of type B trichothecenes. A 50-g amount of sample was placed in a 250-ml Erlenmeyer flask with 22.5 ml of deionised water. The flasks were plugged with cotton, covered with aluminium foil and autoclaved for 20 min at 120°C. The substrate was inoculated with pieces of PDA single-spore cultures of each strain and maintained at 20°C for 3 weeks. The cultures were dried at 45°C for 48 h and then finally ground to powder with a laboratory mill.

In some cases, ground cereal cultures were thoroughly mixed (and thus diluted) in variable ratios with uncontaminated ground cereal samples to provide an approach to the analysis of naturally contaminated samples. Blank samples of cereals were spiked with aliquots of standard solutions of type B trichothecenes and used as substrates for recovery experiments.

2.3. Extraction

Finely ground cereal contaminated samples were thoroughly homogenised for 5 min and extracted for analysis of type B trichothecenes. Five extraction solvents were assayed. These were methanol–water (50:50, v/v), methanol–water (70:30, v/v), acetonitrile water (84:16, v/v), acetonitrile–water (75:25, v/v) and ethyl acetate–acetonitrile–water (77:19:4, v/v/v).

Ground cultures (5 g) were blended in a high speed blender (Ultraturrax T25, IKA, Stauffen, Germany) using a PTFE flask with each solvent (2×25 ml) for 5 min (glass containers did not provide good results). After filtering through Whatman No. 4 filter paper, the filtrate was defatted with hexane (2×10 ml) and extracted with dichloromethane (3×15 ml). The combined dichloromethane extracts were dried over anhydrous sodium sulphate and evaporated to dryness in rotary evaporator prior to clean-up procedure.

2.4. Sample clean-up

2.4.1. Silica and Florisil solid-phase extraction (SPE) cartridges

Using a gas-tight syringe, a Sep-Pak Florisil or silica cartridge (1 g) (both from Waters) was activated with 5 ml of methanol followed by 5 ml of chloroform–methanol (90:10, v/v). The sample residue was dissolved in 2×1.5 ml of chloroform–methanol (90:10, v/v), loaded on the SPE column and forced slowly through the cartridge. The column was washed with 20 ml of chloroform–methanol (90:10, v/v). The 23 ml of eluate was collected into a 100-ml round-bottom flask and evaporated to dryness in a vacuum evaporator at 45°C. The evaporated sample was dissolved twice in 0.5 ml of chloroform and transferred to a 4-ml reaction vial. The vial was placed into water bath kept at 45°C.The solvent was removed under a gentle stream of nitrogen.

2.4.2. Mycosep 225 column

A total of 6 ml of sample extract in acetonitrile–water (84:16, v/v) was placed into the culture tube of a Mycosep 225 column (Romer Labs., Union, MO, USA). The rubber flange end of clean-up column was pushed slowly into culture tube, creating a tight seal between rubber flange and glass wall of the culture tube. As column was pushed farther into tube, the extract was forced carefully through the frit, one-way valve and packing material successively (it should not take <25 s). A 2-ml volume of the purified extract was transferred using a micropipet to a vial. The solvent was evaporated to dryness under a gentle stream of nitrogen.

2.5. Derivatisation for GC determination

The tube must be dry. Residual water destroys the derivatising reagent.

2.5.1. Trimethylsilylation

A 100-μl volume of the derivatisation mixture (see Table 3) was placed into a vial containing the residue. The mixture was shaken on Vortex for 10 s and allowed to react for 5 min at room temperature. Without removing derivatisation agents, 0.5 ml of pentane and 1 ml of water were added. The vial was shaken on Vortex for 2 min and layers were allowed to separate. Top organic layer was transferred to a vial and analysed by GC–ECD.

2.5.2. Fluoroacylation

(a) One ml of DMAP in toluene (2 mg/ml) and 50 μl of HFBA were added to dry sample. The reaction mixture was heated at 60°C for 20 min in a heater

block. After the mixture had cooled, 1 ml of 3% (w/v) aqueous $NaHCO_3$ solution was added and shaken for 10 s on Vortex mixer. The two layers were allowed to separate, the top (toluene) layer was transferred to a GC vial and analysed by GC–ECD.

(b) The residue was trifluoroacetylated with 100 μl of TFAA in the presence or absence of 10 mg of sodium hydrogencarbonate. The vial was capped and heated for 20 min at 60°C in a heater block. After cooling, the excess of derivatising reagent was evaporated under a gentle stream of nitrogen. Then, 500 μl of toluene and 1 ml of redistilled water were added and this mixture was shaken for 1 min. A 300-μl volume of the organic layer was transferred to a GC vial and analysed by GC–ECD.

2.6. GC–ECD analysis

A HP-6890 Plus gas chromatograph (Hewlett-Packard, Avondale, PA, USA) was equipped with a fused-silica capillary column HP-5 (30 m×0.32 mm I.D., 0.25 μm film thickness) and a ^{63}Ni electron-capture detector. The GC–ECD determination was carried out under the following conditions: helium at a flow-rate of 1 ml/min was used as a carrier gas and splitless injection mode was used. A 1-μl volume of the solution was injected. The temperature of the splitless injection port was 250°C and the temperature of the detector was 300°C. The column temperature program was: 80°C held for 1 min, 40°C/min to 160°C held for 3 min, 4°C/min to 240°C held for 2 min and then 30°C/min to 270°C held for 5 min.

2.7. HPLC procedure

2.7.1. Derivatisation with coumarin-3-carbonyl chloride

The procedure has been previously described [39]. A 10-μl volume of DMAP solution in toluene (6.5 mg/ml) was added to a derivatization vial containing evaporated samples or standards. A 10-μl amount of the coumarin reagent was added. The mixture was heated at 80°C for 20 min in a heater block. Then it was cooled in ice water and cleaned-up by liquid–liquid extraction between toluene and 0.05 *M* dihydrogenphosphate buffer, pH 5.5. The cooled reaction mixture was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved with 75 μl of acetonitrile–water (65:35, v/v), acidified with acetic acid to reach a final concentration of 0.75% (v/v) (HPLC mobile phase). The solution was filtered through a 0.20-μm filter and 20 μl were injected into the liquid chromatograph.

2.7.2. HPLC analysis

For fluorescence detection (FL), type B trichothecene derivatives were analysed by liquid chromatography. The HPLC system consisted of a Waters 600 pump and a Waters 474 scanning fluorescence detector. Signals were processed with Millennium software on a Digital Celebris 590 PC. Chromatographic separations were performed on a stainless steel LiChrospher 100 C_{18} reversed-phase column (250×4 mm, 5 μm particle size) connected to a guard column (4×4 mm, 5 μm particle size) filled with the same phase. The column was kept at room temperature. The mobile phase consisted of acetonitrile–water (65:35, v/v), acidified with acetic acid to reach a final concentration of 0.75% (v/v), at a flow-rate of 1.0 ml/min. The mobile phase was degassed by passing through a vacuum-degassing device (Waters). The excitation and emission wavelengths of the fluorometer were set at 292 and 425 nm, respectively, with slit width of 18 nm.

For UV detection, the same equipment was used, with a Waters 996 photodiode array detection (DAD) system. The mobile phase was water–acetonitrile (90:10, v/v) at 1 ml/min and chromatograms were recorded routinely at 221 nm.

2.8. Recovery experiments

Recovery experiments were carried out on wheat, rice and corn spiked with different levels of trichothecenes. To 5 g of finely ground sample in a flask, 100 μl of a type B trichothecene mixture in chloroform were spread over the surface. The flask was shaken manually to distribute the added standards as evenly as possible. The sample was left open at room temperature for 2 h. Any remaining chloroform was evaporated under a stream of nitrogen. The samples were analysed according to the described methodology.

3. Results and discussion

3.1. Extraction solvents

Several extraction solvent mixtures in different volumetric ratios have been employed for extraction of grain samples in published studies [9–18].

In any case, due to the bulk coextractives typically contained in crude extracts obtained by relatively polar solvent mixtures, great demands were placed both on the efficiency of the clean-up step and on the performance of the chromatographic separation, especially when relatively non-specific detection techniques were employed.

Five extraction solvents were investigated to choose the best of them. To perform these studies, ground cereal cultures (corn, rice and wheat) of strain FG6 were assayed and all extracts were injected into the GC–ECD system under the same conditions previous derivatisation with HFBA. The results of the comparative study are shown in Table 1. The mixture acetonitrile–water (84:16, v/v) seems to be the most suitable for extraction of type B trichothecenes from corn, rice or wheat samples, although the ratio (75:25, v/v), as described by Tanaka et al. [18], also provided satisfactory recoveries. Both methanol–water mixtures provided similar yields, but not so acceptable as acetonitrile–water mixtures because more impurities were extracted. The use of ethyl acetate–acetonitrile–water (77:19:4, v/v/v) provided the best extraction data for NIV and 3-AcDON, but effectiveness for DON was very low (Table 1).

The use of the acetonitrile–water (84:16, v/v) mixture for extraction of type B trichothecenes provided the best extracting efficiency from tested solvent mixtures as indicated above (Table 1).

3.2. Clean-up procedure effectiveness

Ground corn cultures, previously tested for presence of type B trichothecenes, were extracted with acetonitrile–water (84:16, v/v). The extracts were purified by the way indicated in Section 2. Several problems were found when silica or Florisil cartridges were used. Low effectiveness was obtained, especially for NIV, due to the low solubility of this polar trichothecene in the less polar solvents used with these cartridges. The high polarity of NIV (due to its four hydroxyl groups) also favours the interaction with active groups of silica (Table 2). Moreover, different evaporation and dissolution steps are needed when using these procedures, critical steps, which are time-consuming and may cause significant losses of toxins.

The recoveries obtained by the clean-up procedures are shown in Table 2. Although efficient removal of impurities was obtained with both silica and Florisil cartridges (colorless solutions, clean chromatograms), lower recoveries were obtained with the last one. Similar problems were found by Weingaertner et al. [40].

The MycoSep 225 column is more simple to be used and provides a faster clean-up method, giving chromatograms comparable to methods consisting of several purification steps. Only one evaporation step, prior to derivatisation, is required. Weingaertner et al. [40] and Romer [41] pointed to potential binding of analytes to active sites of cartridge. Good recoveries for DON, NIV and 3-AcDON, when comparing with

Table 1
Effectiveness of the extraction solvent for type B trichothecenes in contaminated corn by GC–ECD with HFBA derivatisation ($n=3$)

Solvent	Type B trichothecenes					
	NIV		DON		3-AcDON	
	Peak area (μV s)	RSD (%)	Peak area (μV s)	RSD (%)	Peak area (μV s)	RSD (%)
Methanol–water (50:50)	26 298	6.5	64 807	5.1	4332	10.3
Methanol–water (70:30)	23 541	5.9	63 489	8.3	11 236	9.6
Acetonitrile–water (84:16)	38 344	7.2	106 754	3.9	12 489	5.4
Acetonitrile–water (75:25)	39 455	4.9	70 887	6.7	21 846	6.8
Ethyl acetate–acetonitrile–water (77:19:4)	47 654	8.4	34 285	7.2	41 660	8.5

Table 2
Influence of the clean-up procedure on the recovery of type B trichothecenes in contaminated ground corn: acetonitrile–water (84:16, v/v) mixture was used for extraction

Clean-up procedure	Type B trichothecenes					
	DON		NIV		3-AcDON	
	Response (μV s)	Recovery (%)	Response (μV s)	Recovery (%)	Response (μV s)	Recovery (%)
Silica SPE cartridge	63 610	66.5	16 143	31.2	10 173	59.2
Florisil SPE cartridge	76 267	79.7	21 430	41.1	12 342	71.8
MycoSep 225 column	106 754	89.6	39 455	75.6	12 489	72.6

the results provided by silica or Florisil, were obtained when clean-up of grain extract was carried out by the MycoSep 225 column. Only for the more polar NIV the recovery was lower (Table 2).

3.3. Derivatization for GC analysis

Almost all GC methods used for determination of trichothecenes in food and cereals currently are based on prior derivatization of the hydroxyl groups forming trimethylsilyl (TMS), trifluoroacetyl (TFA) or heptafluorobutyryl (HFB) derivatives. The choice of derivatisation reagent depends on the type of trichothecene to be determined and the detector.

3.3.1. Trimethylsilylation

The results of these experiments are shown in Table 3. Fig. 1 shows a typical chromatogram obtained with derivatised standards of type B trichothecenes. DON was efficiently derivatised with all of the derivatisation mixtures except for experiments where pyridine was added to the reaction mixture, provided the major area peaks, higher than those from NIV. When pyridine was present in the derivatisation mixture, 3-AcDON–TMS derivative ECD response was observed as the highest peak in the chromatogram, meanwhile 15-AcDON provided a signal comparable to those from NIV–TMS derivative.

The presence of TMSI in the reaction mixture is essential for an effective derivatisation of the type B trichothecenes [42,43]. In the case of reagent mixtures containing TMSI and TMCS, the presence of BSA seems to be essential to obtain a sufficient response for type B trichothecenes. Without TMCS derivatisation of these mycotoxins almost does not

Table 3
Influence of the derivatisation procedure on the signal of the detector in the GC–ECD system[a]

Derivatisation procedure	Type B trichothecene derivative response (μV s)[b]			
	DON	NIV	3-AcDON	15-AcDON
Trimethylsilylation				
TMSI–TMCS (100:1)	70 082	16 598	23 320	10 931
TMSI–TMCS–pyridine (1:0.2:9)	39 434	16 460	43 038	15 115
TMSI–BSA–TMCS (3:3:2)	99 884	20 664	2083	1874
TMSI–TMCS–chloroform (1:0.2:9)	21 342	10 588	12 680	5417
BSA–TMSI (5:1)	4105	2270	1490	934
TMSI–pyridine (1:2)	2897	2060	3210	1837
Fluoroacylation				
HFBA	344 951	130 011	140 344	–
TFAA+$NaHCO_3$	110 621	38 025	111 004	41 268
TFAA	65 248	39 431	66 023	14 063

[a] TMSI, (trimethylsilyl)imidazole; TMCS, trimethylchlorosilane; BSA, *N,O*-(bistrimethylsilyl)acetamide; HFBA, heptafluorobutyric anhydride; TFAA, trifluoroacetic anhydride.
[b] Average values obtained by triplicate injections.

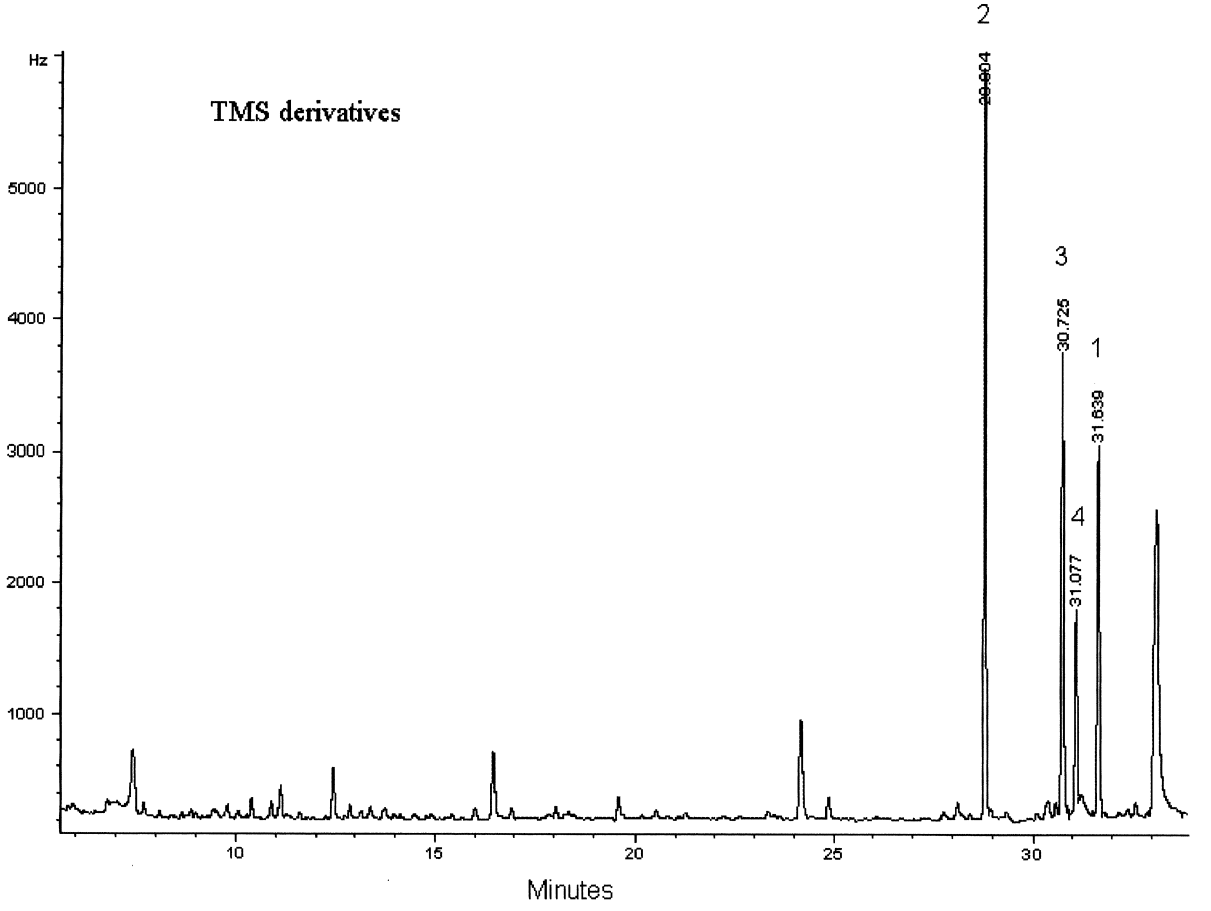

Fig. 1. GC–ECD chromatogram of TMS derivatives of a standard mixture showing the peaks corresponding to the four type B trichothecenes determined. Injected amount, 20 ng. (1) NIV; (2) DON; (3) 3-AcDON; (4) 15-AcDON.

proceed. However, the presence of BSA led to a diminution in the derivatisation efficiency of both 3- and 15-AcDON.

The use of derivatisation mixtures containing TMSI and TCMS (without BSA) led to good derivatisation yields when an excess of TMSI was employed, which is in agreement with the fact that the reaction mixture TMSI–TMCS (100:1) was used in several studies [6,29,33], although the best results, when only DON and NIV were analysed, were obtained with the mixture TMSI–BSA–TMCS (3:3:2) as discussed by Kim et al. [32].

In order to test the reproducibility of the trimethylsilylation methods, peak areas from 10 repeated derivatisation reactions of a standard mixture containing type B trichothecenes were compared. The RSD values were about 15% as derived from the experiments, except when TMSI–TMCS–chloroform (1:0.2:9, v/v) or BSA–TMSI (5:1, v/v) mixtures were used, reaching values higher than 25% (data not shown).

3.3.2. Fluoroacylation

The results of these experiments are shown in Table 3. Fig. 2 shows typical chromatograms of (a) HFBA and (b) TFAA derivatives of type B trichothecenes. The heptafluorobutyryl derivative procedure led to the highest signals for all the type B trichothecenes detected; nevertheless, the peak corresponding to 15-AcDON derivative cannot be observed. Owing to stereochemical structures of DON,

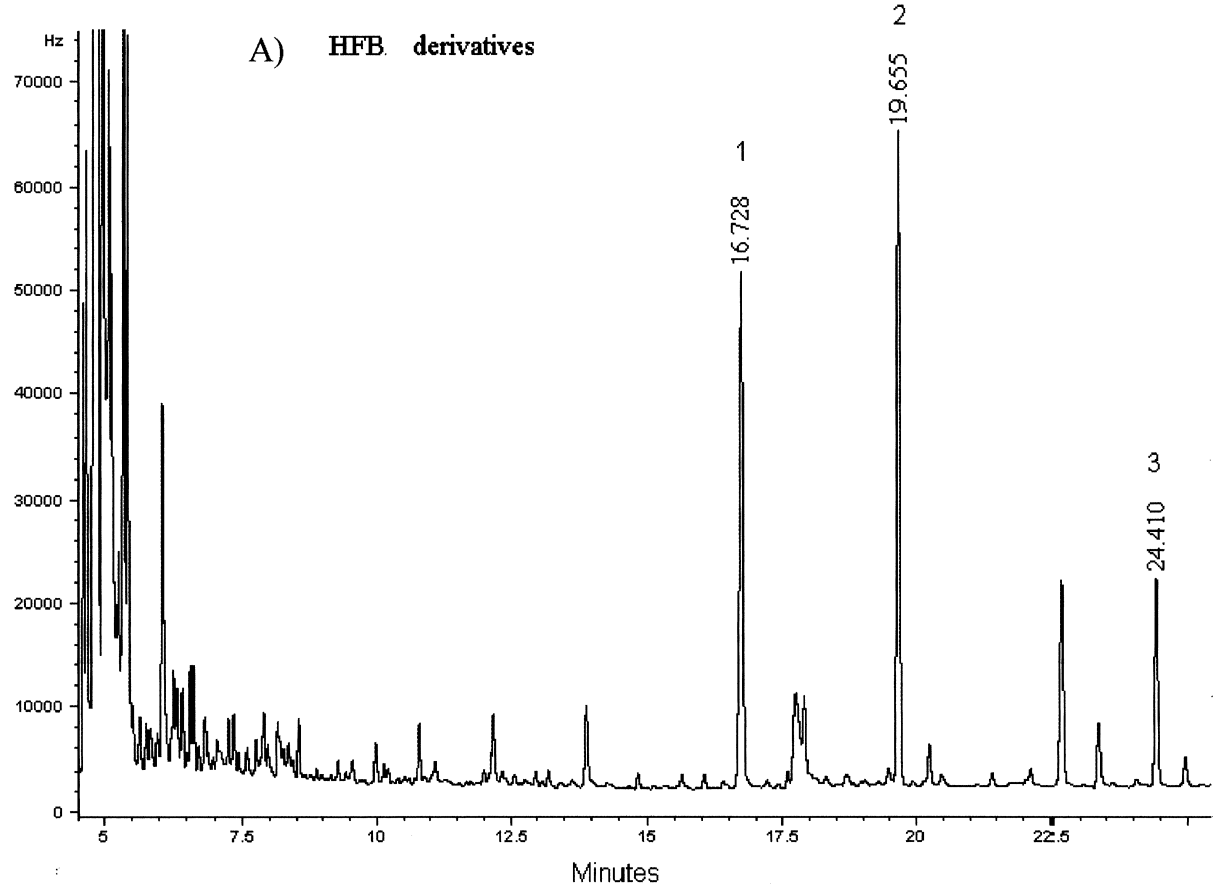

Fig. 2. GC–ECD chromatograms of a standard mixture derivatised with (a) HFBA and (b) TFAA, showing the peaks corresponding to the type B trichothecenes determined. Injected amount, 20 ng. Numbers as in Fig. 1.

3-AcDON and 15-AcDON, this result could be explained. HFBA is unable to react with the hydroxyl group in position 3 of the molecule, derivatising only the groups in positions 7 and 15. The hydroxyl group in C-15 is acetylated in 15-AcDON, preventing the derivatisation of hydroxyl group in C-7 by steric reasons.

TFAA was chosen for practical reasons because the excess of the reagent could easily be removed completely by evaporation. During derivatisation, pyridine or triethylamine was used as acid acceptor [43], but these basic solvents give rise to broad solvent peaks. Therefore, $NaHCO_3$, being a solid acid acceptor, was preferred. As a result, the type B trichothecenes showed much higher peak areas compared with the case where TFAA without the acid acceptor was applied.

In order to test the reproducibility of the fluoroacylation methods, peak areas obtained from a standard mixture containing all type B trichothecenes were compared. RSD values were lower than 10% in all the cases, as derived from 10 replicates.

Comparing both derivatisation strategies, fluoroacylation of mycotoxins provides better results than trimethylsilylation for GC–ECD; among acylation reagents, HFBA provided the highest toxin peak areas, although 15-AcDON was not derivatised. For determination of this toxin, the utilisation of TFAA

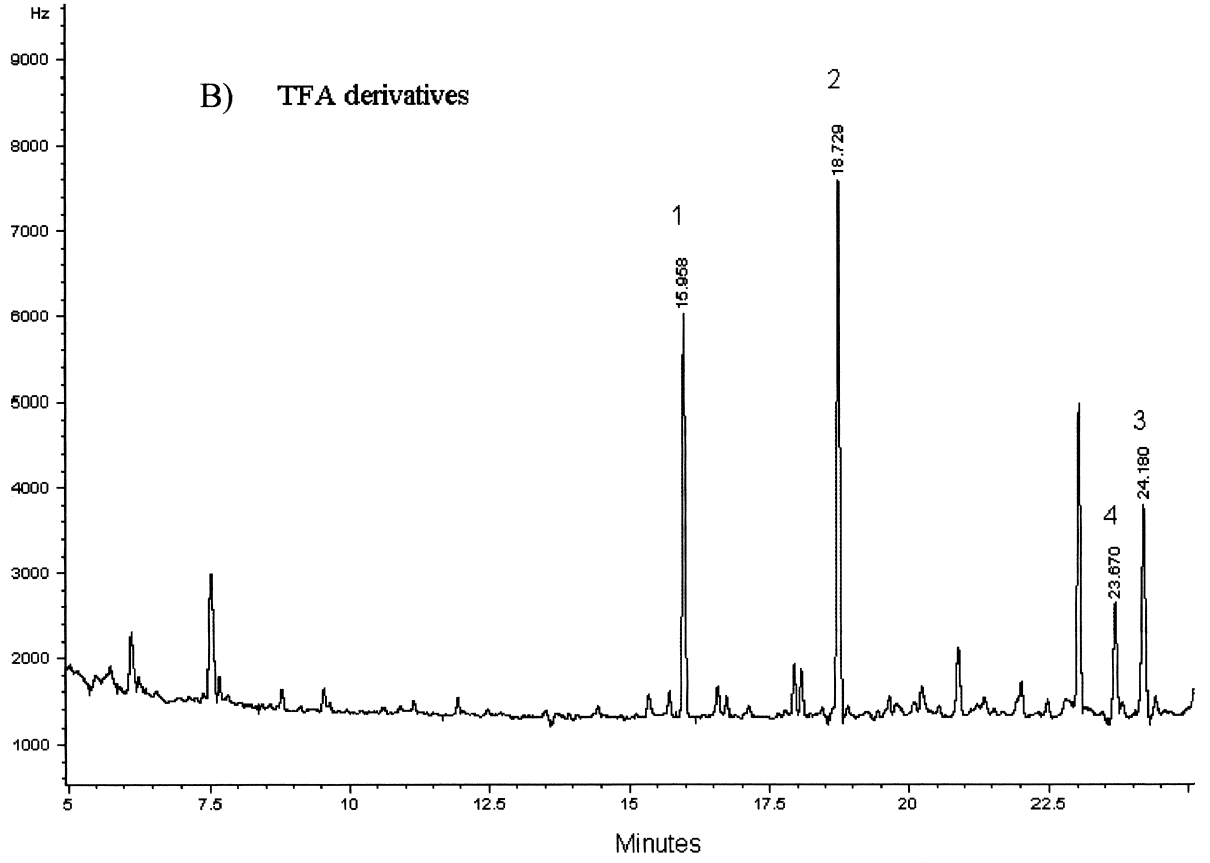

Fig. 2. (*continued*)

reagent with sodium bicarbonate is recommended, giving also acceptable results for the other trichothecenes.

3.4. Derivatization for HPLC–FL analysis

The derivatisation conditions have been previously explained [39]. Fig. 3 shows a typical HPLC–fluorescence detection chromatogram of a standard mixture derivatised with coumarin-3-carbonyl chloride, showing the peaks corresponding to the four type B trichothecenes determined. All the peaks were correctly separated but some interferences from the excess of reactive peaks could be observed because, due to the low response obtained, the clean-up step after derivatisation of trichothecenes was omitted and the excess of reagent appears as a broad, highly fluorescent peak.

The signal from the 3-AcDON derivative was higher than the response from other type B trichothecenes. The DON derivative is shown as a small wide peak. Maybe, the presence of acetyl group esterifying the hydroxyl group at C-3 favours the derivatisation reaction. Response reproducibility of the derivatives was similar to that found for type A trichothecenes [39].

3.5. Evaluation of calibration lines and limits of detection

The limits of detection for both GC–ECD and HPLC procedures were obtained by injections of

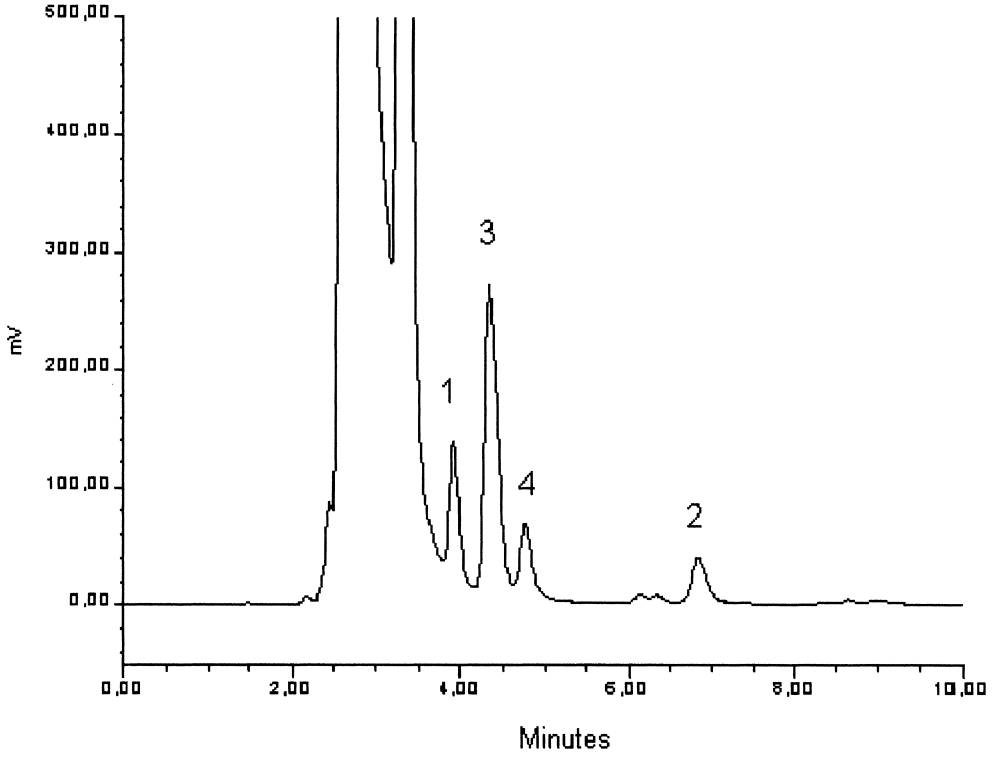

Fig. 3. HPLC–fluorescence detection chromatogram of a standard mixture derivatised with coumarin-3-carbonyl chloride, showing the peaks corresponding to the four type B trichothecenes determined. Injected amount, 10 ng. Numbers as in Fig. 1.

spiked samples and blank samples. For GC–ECD procedures, responses were linear in the range 0.01–100 ng of each type B trichothecene, using both HFB or TFA derivatives, with correlation coefficients higher than 0.999. The derivatisation method was more sensitive for DON in both cases while lower sensitivities were obtained with 3-AcDON and 15-AcDON. The limits of detection (signal-to-noise=3) were 15 pg for DON and NIV, and 25 pg for 3-AcDON with HFBA; meanwhile they were 25 pg for DON, 50 pg for both NIV and 3-AcDON, and 0.3 ng for 15-AcDON with TFA. These data express the amount of trichothecenes (underivatised) in the 1 μl injected into the GC.

For HPLC procedures, the calibration curves were linear in a broader interval, from 0.01 to 500 ng, with correlation coefficients higher than 0.999. DAD provided greater responses for underivatised type B trichothecenes than the fluorescence detector for the respective coumarin-3-carbonyl derivatives. These data could be explained by incomplete derivatisation of type B trichothecenes when coumarin-3-carbonyl chloride was used to obtain fluorescent derivatives (Fig. 3). These data are different from those previously obtained for type A trichothecenes [39] and may be explained by the different polarity of both type of trichothecenes. Coumarin-3-carbonyl chloride reagent is more suitable to be employed for type A trichothecene analysis. The limits of detection were about 5 pg for all type B trichothecenes when photodiode array detection was used, while they were 10 pg for 3-AcDON, 20 pg for NIV and 15-AcDON, and 25 pg for DON when fluorescence detection was used. This method has the advantage to provide a good chromatographic separation of the peaks corresponding to the four analytes but it is necessary to diminish the limits of detection to the same level to that obtained for type A trichothecenes [39]. This is the first time that this derivatisation method is applied to type B trichothecenes.

The mean value of RSD of the areas obtained by injection of 20 ng/μl standard solution of type B trichothecenes 10 times was 5.26% with GC–ECD of HFB derivatives and 6.39% with GC–ECD of TFA derivatives. The reproducibility of the injection was 1.98% (HPLC–DAD) and 4.96% (HPLC–FL). Thus, responses obtained with DAD were the most precise. The better RSD data obtained with HPLC–DAD method could be explained taking into account that trichothecenes were not derivatised by this methodology.

3.6. Choice of the instrumental method

The choice of method depends on the required detection level, matrix and instrumentation available. HPLC can be employed for samples with high levels of type B trichothecenes. Nevertheless, the utilisation of DAD is highly recommended because a derivatisation step is unnecessary, good resolution is reached and lower limits of detection are obtained. For fluorescence detection, clean-up step after derivatisation used for type A trichothecenes was omitted [39] because of the low responses of derivatised toxins, making limits of detection unacceptable. Nevertheless, further experiments will be made in order to optimise the method and decrease the limits of detection for type B trichothecenes.

On the basis of this comparative study, we propose the use of GC–ECD previous derivatisation with HFBA for the determination of DON and NIV. This procedure gives low limits of detection and a high reproducibility and sensitivity for the determination of these toxins. On the other hand, the derivatisation procedure is easy and rapid to carry out. Nevertheless, if acetyl forms of DON are going to be analysed, the TFA derivatisation procedure prior to GC–ECD analysis is recommended.

3.7. Recovery experiments

After the comparative study of extraction solvents and SPE clean-up procedures, the combination that produced the best results was chosen for recovery studies on spiked samples. Samples of ground cereals were spiked with standard solutions of DON, NIV and 3-AcDON. The contamination levels ranged from 0.025 to 12.5 μg/g. After solvent evaporation and thorough mixing, the samples were extracted with acetonitrile–water (84:16, v/v) as described in Section 2. Extracts were purified through MycoSep 225 column and analysed by GC–ECD following previous derivatisation with HFBA as described in Section 2.5.2(a). The results of these assays are shown in Table 4. Recoveries for DON were 75.7–96.8% in corn, 76.5–96.2% in rice, and 72.9–94.6% in wheat. Recoveries for NIV were 59.4–72.2% in corn, 57.2–70.9% in rice, and 53.6–69.6% in wheat.

3.8. Analysis of cereal cultures of Fusarium

The optimised method was applied to the analysis of type B trichothecenes in corn, wheat and rice cultures inoculated with three strains of *Fusarium* (see Section 2). The results are listed in Table 5. Fig. 4 shows the GC–ECD chromatogram of a corn sample inoculated with FG1 strain and incubated at 15°C during 3 weeks. As some authors have re-

Table 4
Percentage of recovery for type B trichothecenes in spiked corn, rice and wheat: analytical method, GC–ECD of HFB derivatives

Spiking level (μg/g)	Corn			Rice			Wheat		
	DON (%)	NIV (%)	3-AcDON (%)	DON (%)	NIV (%)	3-AcDON (%)	DON (%)	NIV (%)	3-AcDON (%)
12.5	75.7	59.4	60.3	76.5	57.2	61.2	72.9	53.6	57.9
5	78.2	60.2	68.8	81.7	60.2	64.0	75.4	57.2	59.8
2.5	84.9	63.9	71.6	83.0	63.7	69.4	80.3	59.3	63.8
1.25	87.2	67.7	73.5	88.2	65.9	72.8	84.7	64.0	69.4
0.5	89.2	71.5	74.5	91.8	69.0	74.3	89.1	66.7	72.0
0.25	91.4	72.1	75.4	92.5	70.2	75.7	91.9	69.5	74.4
0.025	96.8	72.2	75.9	96.2	70.9	76.8	94.6	69.6	75.4

Table 5
Type B trichothecenes levels found by GC–ECD and HFBA derivatisation on cereal cultures of three selected *Fusarium* strains incubated at 15°C for 3 weeks in triplicate[a]

Cereal/ *Fusarium* strain	Type B trichothecenes					
	DON		NIV		3-AcDON	
	μg/g culture	RSD (%)	μg/g culture	RSD (%)	μg/g culture	RSD (%)
Corn/FG1	0.359	7.9	1.893	5.2	1.2	3.9
Rice/FG1	1.101	4.3	0.074	8.9	2.76	4.1
Rice/FC1	ND	–	ND	–	ND	–
Rice/FG6	2.685	3.8	8.267	1.6	0.124	6.4
Wheat/FC1	ND	–	ND	–	ND	–
Wheat/FG6	tr.	–	2.859	3.1	tr.	–

[a] tr, traces (<2×limit of detection); ND, not detected.

ported, our data show that *F. culmorum* is unable to produce type B trichothecenes, although more experiments will be made to confirm this affirmation. Both strains of *F. graminearum* can produce these mycotoxins. An approach to the analysis of naturally contaminated cereals was simulated by the addition of cultures with trichothecene content to uncontaminated samples at low ratios (usually, about 10%). The entire procedure worked satisfactorily as the levels found for the different type B trichothecenes agreed within 10% with the predicted concentrations in the contaminated samples. Therefore, the opti-

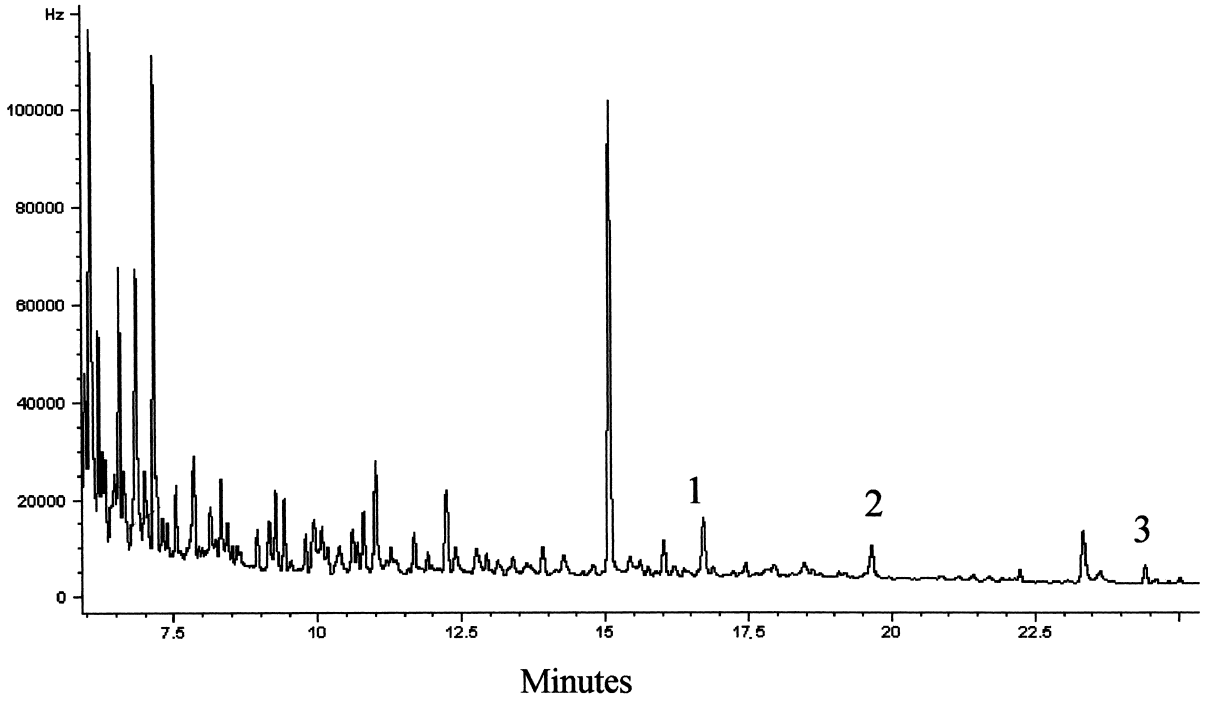

Fig. 4. GC–ECD chromatogram of a corn sample inoculated with FG1 strain and incubated at 15°C during 3 weeks. Concentration of each type B trichothecene is shown in Table 5. Numbers as in Fig. 1.

mised GC–ECD method using HFBA derivatisation is applicable to the quantitative analysis of these mycotoxins in cereal samples provided their content is higher than 0.025 μg/g (3–5×limit of detection). These results were consistent with those previously published by other authors [44–46].

4. Conclusions

In order to select a reliable, accurate and rapid chromatographic method to analyse for type B trichothecenes in cereal cultures and maybe in naturally contaminated grains, five extraction solvents, three clean-up procedures an GC of TMS or fluoroacyl derivatives and HPLC of underivatised toxins or coumarin-3-carbonyl chloride derivatives were compared. As far as we know, this last derivatisation procedure is applied here for the first time to DON, NIV, 3 and 15-AcDON. The best extraction solvent consisted of a mixture of acetonitrile–water (84:16, v/v), which provided higher extraction efficiency than other mixtures. A simple clean-up with MycoSep 225 column provided better trichothecene recoveries than silica or Florisil SPE cartridges. For GC–ECD analysis, fluoroacylation of these mycotoxins provided better results than trimethylsilylation, as expected. Among acylation reagents, HFBA provided the lowest limits of detection, although 15-AcDON was not derivatised. For determination of this toxin, the utilisation of TFAA reagent with sodium hydrogen carbonate is recommended, giving also acceptable results for the other trichothecenes. When HPLC was used for analysis of type B trichothecenes, photodiode array detection (at 221 nm) seems preferable instead of fluorescence detection because lower limits of detection are reached and no derivatisation step is necessary. However, further experiments will be made regarding the synthesis and purification of coumarin-3-carbonyl fluorescent derivatives of type B trichothecene in order to improve this new method. The optimised method was applied to the determination of DON, NIV and 3-AcDON in corn, wheat and rice cultures of *F. culmorum*, and *F. graminearum* strains, showing a broad range for these toxins. Based on previous assays with cereal samples mixed with cultures containing type B trichothecenes at very low mixing rates the method is applicable to naturally contaminated grains.

Acknowledgements

Financial support from the Spanish Comision Interministerial de Ciencia y Tecnologia (Project Ref. ALI98-0850) is gratefully acknowledged.

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